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ANTHRAX TOXIN

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Anthrax toxin is a key virulence factor of Bacillus anthracis. The three protein components of the toxin have been purified and shown to have similar molecular weights: protective antigen (PA), 85,000; lethal factor (LF), 83,000; edema factor (EF), 89,000. The edema factor acts by reusing the concentration of cAMP in animal cells, and subsequently EF was found to be a calmodulin-dependent adenylate cyclase. The similarity of EF to Bordetella pertussis and eukaryotic cyclases suggests that the anthrax EF gene may have originated in animals. The lethal factor causes death in rats in as little as (cont. on back)

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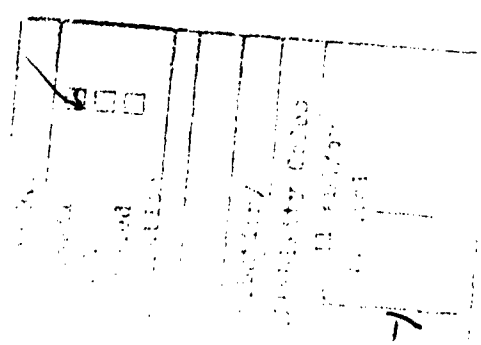
38 minutes. No cultured cells are known which are rapidly damaged by LF.

Anthrax Toxin

Anthrax is a serious, frequently fatal disease usually confined to herbivores. Humans, however, can be infected through contact with diseased animals, as in recent outbreaks in Zimbabwe, where more than 3,000 cases were recorded (4). The study of anthrax by early microbiologists established several of the fundamental concepts of pathogenic microbiology. Protective measures developed by early workers largely controlled the disease; one result of this was decreased interest in its study. Thus, anthrax has been largely ignored during a period in which powerful methods of biochemical and genetic analysis have successfully elucidated the molecular processes by which other pathogens engender disease. Only within the last three to four years have extensive studies on anthrax resumed. These are discussed in two other presentations in this symposium (Thorne; Mikesell and Vodkin) and in this paper, which describes the current status of research on the structure and mechanism of action of anthrax toxin.

Bail and Weil (1) presented evidence for "aggressins" in anthrax-infected guinea pigs, but it was Smith and coworkers (11, 12) who first directly demonstrated the existance of an anthrax toxin. Subsequent work revealed that Bacillus anthracis secretes three separate proteins which are individually non-toxic, but act in binary combinations to produce two distinct toxic responses, edema in skin and lethality. The American nomenclature and active combinations of these proteins are shown in Fig. 1. The central toxin component was recognized to be the same as the immunogen first identified by Gladstone (6), and it has retained the original name, protective antigen



(PA). The ability of antibodies against PA to protect against infection is consistent with the central role toxin plays in anthrax infection and with the fact that such antibodies are predicted to neutralize both toxic activities. PA is the major component in the current licensed human anthrax vaccine. The subject of anthrax vaccines has recently been reviewed (7).

Work in this laboratory began with efforts to produce amounts of the anthrax toxin components which would be sufficient to support a varied and comprehensive research program. The unencapsulated, toxigenic Sterne strain was grown in a 20-L and later a 50-L fermenter, using the synthetic R medium (10). Culture supernatants were diluted and the proteins adsorbed to DEAE-cellulose. Toxin was eluted with NaCl, precipitated with $(NH_4)_2SO_4$, dialyzed, and chromatographed on hydroxyapatite (9). The three components eluted in distinct peaks, each of which was rechromatographed on DEAE-Trisacryl M (LKB) to obtain proteins which were at least 90% pure as determined by electrophoresis on SDS gels. The molecular weights estimated from migration on SDS gels are: PA, 85,000; LF, 83,000; and EF, 89,000. Each protein was used to raise antisera in rabbits and goats; more recently, mouse monoclonal antibodies to each component have been produced. Enzyme linked immunosorbent (14), rocket immunoelectrophoretic (5), and radial immunodiffusion assays have been developed for the individual toxin components using the polyclonal sera. These assays have been used to detect and quantitate toxin components during their purification. Sterne strain culture supernatants were found to contain PA, LF, and EF in concentrations of about 15, 3, and 1 µg/ml, respectively. Yields of purified components from a 20-L fermenter have averaged 50 mg PA, 15 mg LF, and 3 mg EF, well below those expected from the initial concentrations. It can be expected that innovations in the recovery

and purification processes will greatly increase yields. The close similarity in molecular weights was rather unexpected, and raised the possibility that the three components might be related. However, analyses with the polyclonal sera do not reveal any serological relatedness among the components.

The ability to produce adequate amounts of toxin components made it possible to explore their mechanisms of action. This effort focused initially on EF because it seemed possible that this component, like cholera toxin, might cause edema in skin through elevation of cellular cAMP concentrations in the affected tissues. This hypothesis was confirmed when it was shown that Chinese hamster ovary (CHO) cells elongate when treated with PA and EF, and that elongation reflected elevated cAMP concentrations (Table 1). A more extensive analysis of the kinetics of the response in CHO cells revealed that cAMP concentrations rose without a lag to very high values and then decreased when toxin was removed from the medium. This behavior differed from that seen in cells exposed to cholera toxin, where cellular cAMP levels remain elevated upon toxin removal. Studies in CHO cell membrane preparations made it clear that EF was not activating eukaryotic adenylate cyclase, but that EF was itself an adenylate cyclase (8). A unique feature of this bacterial adenylate cyclase revealed in subsequent experiments is the absolute dependence of its enzymatic activity on the presence of calmodulin, a eukaryotic calcium-binding protein (9). These properties suggested a simple model of EF action in which PA binds to cell-surface receptors to form an uptake system by which EF gains access to the target cell cytoplasm. Once in contact with the cytoplasm, EF binds calmodulin and becomes enzymatically active, converting ATP to cAMP. The resulting effects are the same as those caused by cholera toxin. The lethal factor (LF) component of toxin is postulated to enter cells via the

same receptor system, a conclusion based on the competitive actions of LF and EF (8).

The identification of a bacterial adenylate cyclase which enters eukaroytic cells and raises cAMP concentrations was unexpected and appeared unique, but it has turned out to be only part of a larger story in pathogenic mechanisms. As this work was being done, others demonstrated that Bordetella pertussis adenylate cyclase can also enter eukaryotic cells (3). This enzyme had previously been shown to be calmodulin dependent (15), but no role for it in pathogenesis was known. It remains difficult to purify the pertussis enzyme, and no direct evidence is available on how it enters cells. Comparative studies on the anthrax and pertussis cyclases may lead to a better understanding of how this newly recognized type of bacterial toxin contributes to pathogenesis.

The existence of calmodulin-dependent adenylate cyclases in two unrelated bacterial species raised the question of their genetic origins. In B. anthracis there seems to be no evidence for existance of an adenylate cyclase involved in normal metabolic systems that might qualify as an ancestral protein. Indeed, repeated attempts to detect either cAMP or adenylate cyclase in the Bacillus genus have been unsuccessful (2). Furthermore, since calmodulin is not present in prokaryotes, these adenylate cyclases would be non-functional and thereby superfluous in bacterial metabolism. Several of their properties, particularly the dependence on calmodulin, suggest that the two bacterial adenylate cyclases are more closely related to eukaryotic enzymes and may be the products of genes that were acquired from eukaryotes. The validity of this speculation will only be known once the genes for the enzymes are sequenced and examined for homology.

The apparent similarity of EF to eukaryotic adenylate cyclase prompted an examination of its enzymatic properties (9). The purified enzyme was quite stable in adenylate cyclase assay reaction mixtures, with cAMP production continuing at a constant rate for at least 2 hours. Unlike some preparations of the pertussis cyclase, EF is absolutely dependent on the presence of calmodulin (Fig. 2). Half-maximal activation is achieved at about 2 nM calmodulin. As expected for a calmodulin-dependent process, Ca^{+2} was also required, and chelation of Ca^{+2} by EGTA was inhibitory. The effect of Ca^{+2} is actually biphasic, stimulatory at low concentrations (10-100 μm) and inhibitory at high concentrations (1 mM), a behavior also displayed by bovine brain adenylate cyclase. Inhibition by Ca^{+2} is eliminated if the divalent ion Mg^{+2} is replaced by Mn^{+2}. This behavior again matches that of eukaroytic cyclases. The specific activity of EF (V_{max} = 1.2 mmoles min^{-1} mg^{-1}) exceeds that of previously characterized adenylate cyclases, and corresponds to a turnover number of 1800/sec, a value characteristic of a highly efficient enzyme.

In contrast to the success in characterizing EF, progress in understanding the mechanism of LF has been slow. The rat lethality bioassay developed many years ago remains the best characterized response to LF. A typical experiment is shown in Table 2. Neither PA nor LF alone is toxic, but the combination causes severe pulmonary edema and death in 40-60 minutes. The assay is remarkably precise; duplicate determinations yield times to death (TTD) differing by only 1-2 minutes. An extensive analysis of dose responses to each toxin component was performed (5). Reciprocal plots of TTD versus dose of each toxin component were linear, and extrapolated to a minimum TTD of 38 minutes. This is apparently the time required for toxin to bind and enter sensitive cells and for the sequence of biochemical changes leading to

pulmonary edema to occur. This assay was also used to test the hypothesis that LF and EF compete for binding to receptors. Addition of EF to lethal combinations of PA and LF did indeed extend the TTD (Table 2).

Although useful for quantitating PA and LF, the rat bioassay is not likely to be an appropriate system for studying the cellular and molecular mechanisms of action of LF. Therefore, a survey of primary and established cell cultures was performed to identify cells sensitive to LF. The general finding was that confluent monolayers were refractory and showed no cytopathic effects. If cells were plated at very low density and then exposed to toxin for extended periods, inhibition of growth was observed in some cell lines. Taken together, these results suggest that LF does enter most types of eukaryotic cells, and that the cellular activity affected by LF may be one involved in growth or maintenance of cellular structures. The rapid death of toxin-treated rats clearly rules out inhibition of macromolecular syntheses as the mechanism of action, and suggests that there are certain essential cell types uniquely sensitive to toxin that remain to be identified. In this regard, the recent demonstration at this Institute that macrophages are killed within several hours by the combination of PA and LF (A. M. Friedlander, personal communication) indicates that a useful cell model for studying LF action has been identified.

Future studies on the structure of the toxin components will benefit from the recent cloning of the genes coding for PA (14) and LF (D. Robertson and S. Leppla, manuscript in preparation). This work will make it feasible to construct B. anthracis strains producing subsets of the three toxin components. Such strains may hold promise as live vaccines acceptable for use in humans.

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Table 1. cAMP concentrations in toxin-treated Chinese hamster ovary cells[1]

Toxin	Concentration (µg/ml)	cAMP per 35 mm dish (pmole)
None	----	2.8
Cholera toxin	0.1	16.0
PA	1.0	3.4
EF	1.0	3.4
LF	5.0	2.6
PA + EF	as above	150.0
PA + EF + LF	as above	7.0

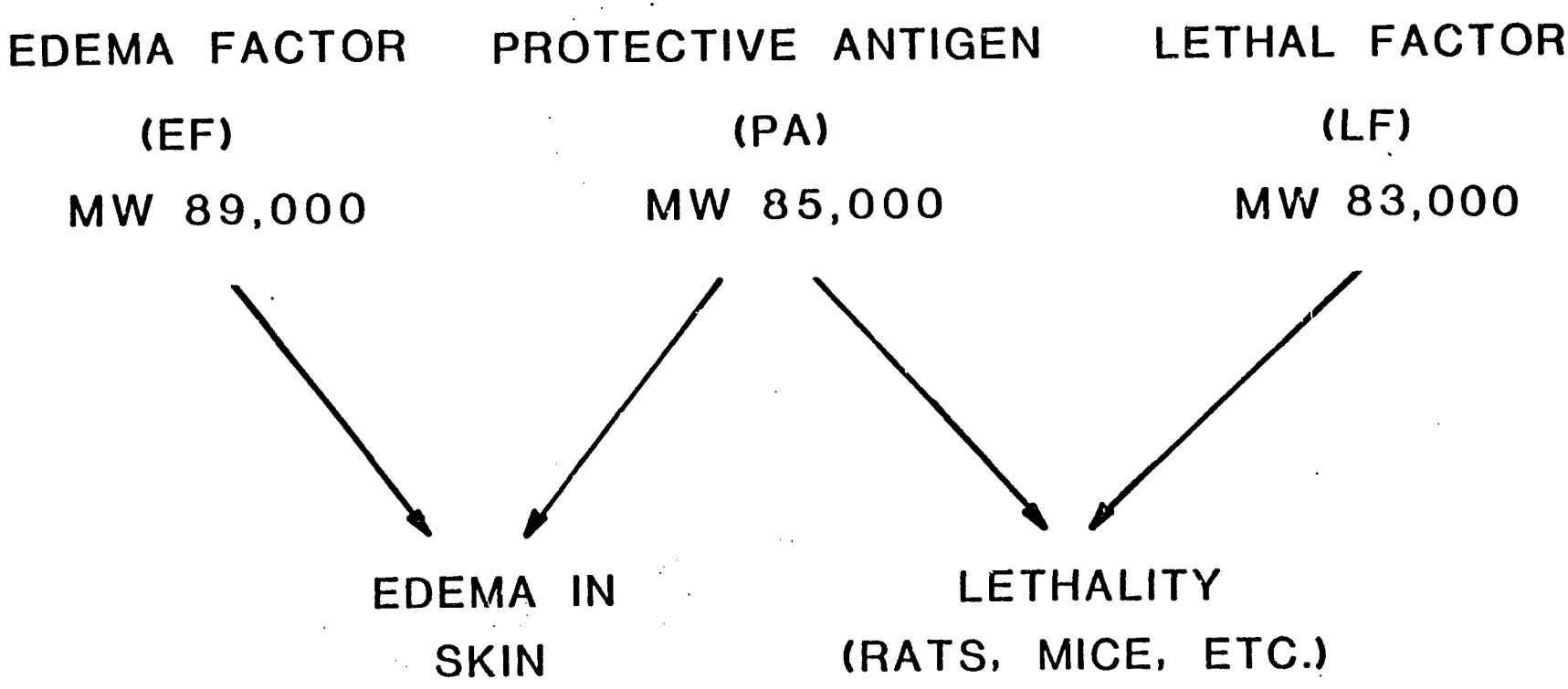
[1]Confluent monolayers of cells were treated with toxin as indicated for two hours. Monolayers were washed, extracted with 0.1 m HCl, and cAMP was measured by radioimmunoassay.

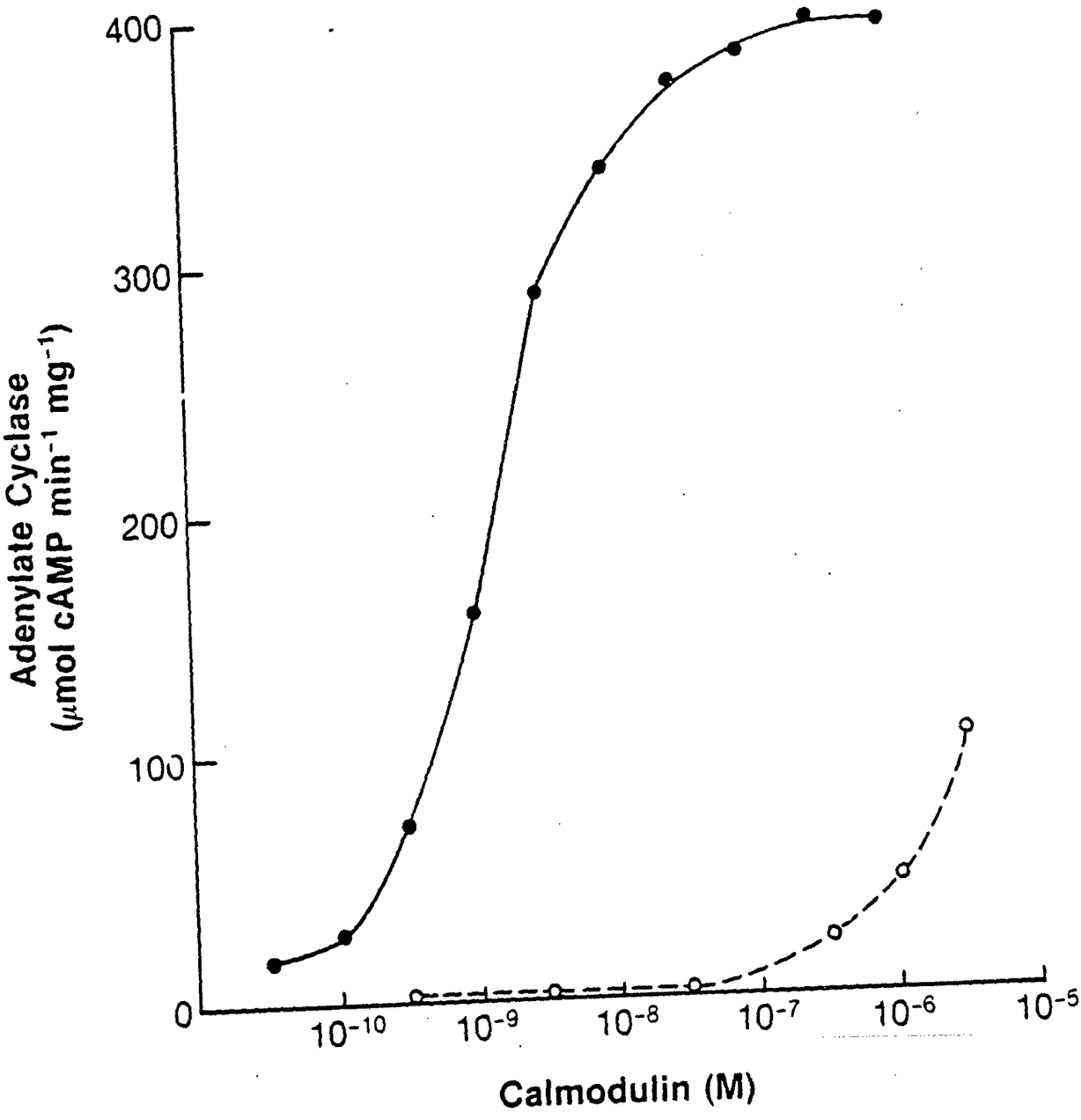
Table 2. Lethal response of Fischer 344 rats to anthrax toxin components

Toxin components injected (µg)			Time to death (min)
PA	LF	EF	
100	0	0	survived
60	1.5	0	90
60	2.5	0	66
60	4.5	0	52
60	8.0	0	42
0	100.0	0	survived
5	8.0	0	70
5	8.0	8	79
5	8.0	40	92

Fig. 1. Anthrax toxin components and their toxic combinations.

Fig. 2. Requirement of edema factor adenylate cyclase activity for calmodulin and calcium. Reactions contained 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5, 0.05 mM cAMP, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.5 mM ^{32}P-ATP (6×10^4 cpm/assay), 10 mM $MgCl_2$, 0.1 mM EDTA, 4 ng/ml EF, and either: (o) 50 μM Ca^{+2}, or (o) 2.7 mM EGTA. Reactions were incubated 30 min at 23°C, and ^{32}P- cAMP was isolated by chromatography on Dowex and alumina columns (9).


EDEMA FACTOR
(EF)
MW 89,000
PROTECTIVE ANTIGEN
(PA)
MW 85,000
LETHAL FACTOR
(LF)
MW 83,000
EDEMA IN
SKIN
LETHALITY
(RATS, MICE, ETC.)


400
300
200
100
0
Adenylate Cyclase
(μmol cAMP min⁻¹ mg⁻¹)
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
Calmodulin (M)